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The H4K20 methyltransferase Kmt5 is involved in secondary metabolism and stress response in phytopathogenic *Fusarium* species

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ABSTRACT

Fusarium fujikuroi and *Fusarium graminearum* are agronomically important plant pathogens, both infecting important staple food plants and thus leading to huge economic losses worldwide. *F. fujikuroi* belongs to the *Fusarium fujikuroi* species complex (FFSC) and causes bakanae disease on rice, whereas *F. graminearum*, a member of the *Fusarium graminearum* species complex (FGSC), is the causal agent of Fusarium Head Blight (FHB) disease on wheat, barley and maize. In recent years, the importance of chromatin regulation became evident in the plant-pathogen interaction. Several processes, including posttranslational modifications of histones, have been described as regulators of virulence and the biosynthesis of secondary metabolites. In this study, we have functionally characterised methylation of lysine 20 histone 4 (H4K20me) in both *Fusarium* species. We identified the respective genes solely responsible for H4K20 mono-, di- and trimethylation in *F. fujikuroi* (*FfKMT5*) and *F. graminearum* (*FgKMT5*). We show that loss of Kmt5 affects colony growth in *F. graminearum* while this is not the case for *F. fujikuroi*. Similarly, FgKmt5 is required for full virulence in *F. graminearum* as Δ*fgkmt5* is hypovirulent on wheat, whereas the *F. fujikuroi* Δ*ffkmt5* strain did not deviate from the wild type during rice infection. Lack of Kmt5 had distinct effects on the secondary metabolism in both plant pathogens with the most pronounced effects on fusarin biosynthesis in *F. fujikuroi* and zearalenone biosynthesis in *F. graminearum*. Next to this, loss of Kmt5 resulted in an increased tolerance towards oxidative and osmotic stress in both species.

1. Introduction

Plant pathogenic fungi are the cause of severe diseases on agricultural crops and thus, lead to huge economic losses each year (Fausto et al., 2019). Apart from significant reduction in yield, the potential contamination of food and feed by potent mycotoxins accumulating during infection represents a severe problem (Fisher et al., 2012; Zain, 2011). The two *Fusarium* species *Fusarium fujikuroi* and *Fusarium graminearum* are the causal agents of bakanae disease of rice (Wiemann et al., 2013) and Fusarium Head Blight (FHB) disease of wheat and barley (Kazan et al., 2012), respectively, and they are occurring worldwide. Both fungi have developed highly efficient infection strategies coupled with the production of secondary metabolites (SMs), including potent mycotoxins to ensure a successful colonisation of the host (Brown et al., 2010; Goswami and Kistler, 2004; Munkvold, 2017; Niehaus et al., 2017; Wiemann et al., 2013). As SM biosynthesis is energy-consuming, SM gene expression is tightly regulated and only initiated under certain conditions. Generally, genes involved in SM biosynthesis are physically linked and organised in clusters (Keller and Hohn, 1997), which facilitates co-regulation, for example by chromatin-based mechanisms (Collemare and Seidl, 2019; Pfannenstiel and Keller, 2019). Here, posttranslational modifications (PTMs) of histone proteins are well-known for controlling gene expression in response to environmental or developmental signals as they have the capacity to define the degree of compaction from an 'open' transcriptionally active euchromatin to 'silent' transcriptionally inactive heterochromatin (Bannister and Kouzarides, 2011). Thus, a certain combination of histone PTMs can strongly influence the accessibility of chromatin and further provide a recognition platform for other chromatin-modifying enzymes, remodelling complexes and transcription factors, which consequently regulate

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transcription of underlying genes (Jenuwein, 2002; Kouzarides, 2007; Wood et al., 2005). While many different histone PTMs are known to date, only a few have been characterised in detail thus far. Histone acetylation and methylation were the first PTMs to be discovered (Allfrey et al., 1964) and since then continue to be the most well characterised histone PTMs. Acetylation is associated with transcriptional activation, whereas methylation has a bivalent role in transcriptional activation and repression, depending on the modified histone residue as well as the degree of methylation. For example, methylation of histone 3 lysine 4 (H3K4) is a hallmark for transcriptionally active euchromatin (Li et al., 2008; Pekowska et al., 2011; Pinskaya and Morillon, 2009), while H3K27me3 and H3K9me3 are hallmarks of facultative and constitutive heterochromatin, respectively (Freitag, 2017; Ridenour et al., 2020; Selker, 2017). A less characterised histone PTM in fungi is H4K20 trimethylation that is considered a silencing mark in higher eukaryotes (Kourmouli et al., 2004; Schotta et al., 2004). Methylation of H4K20 is established by members of the KMT5 family, containing the catalytically conserved SET (Su(var)3–9, Enhancer-of-zeste and Trithorax) domain (Jones and Gelbart, 1993). In vertebrates KMT5 A (PR-Set7/Set8) establishes the mono-methylation, whereas the two enzymes KMT5 B and KMT5 C (Suv4-20 h1/2) catalyse di- and trimethylation of H4K20 (Fang et al., 2002; Nishioka et al., 2002; Schotta et al., 2004). In contrast, the fission yeast *Schizosacharomyces pombe* possesses only one KMT5 orthologue i.e., Set9, which is solely responsible for all three types of H4K20 methylation (Sanders et al., 2004). While H4K20 methylation has been associated with many biological functions in vertebrates (ensuring genome integrity (Beck et al., 2012; Li et al., 2016; Oda et al., 2009), DNA damage repair (H4K20me2) (Chitale and Richly, 2018; Paquin and Howlett, 2018) and chromatin compaction (H4K20me3) (Schotta et al., 2004; Sims et al., 2006; Wang and Jia, 2009)), studies in *S. pombe* demonstrated that H4K20 methylation does not affect gene expression or heterochromatin function (Sanders et al., 2004). Rather, it seems H4K20me is limited to a function in DNA damage response (Sanders et al., 2004; Wang et al., 2009). However, almost no knowledge exists about H4K20 methylation in filamentous fungi, except for *Magnaporthe oryzae*, where MoKmt5 was shown to be the sole histone methyltransferase involved in H4K20me3 (Pham et al., 2015).

In this study, we set out to get a better understanding on the role KMT5 homologues play in the two plant pathogens *F. fujikuroi* and *F. graminearum*. For this, the KMT5 homologues were identified and characterised with regard to growth, asexual development, stress response, secondary metabolism and virulence. The KMT5 homologues are solely responsible for all methylation degrees of H4K20me in both *Fusarium* spp. Loss of Kmt5 results in increased stress tolerance on media supplemented with oxidative and osmotic agents in both species, while overexpression of *KMT5* largely reversed this phenotype. Next to this, H4K20me is crucial for wild type-like secondary metabolism in both fungi. Notably, some observed phenotypes such as reduced hyphal growth and attenuated virulence were only attributed to one of the fusaria, suggesting additional species-specific functions of Kmt5.

2. Materials and methods

2.1. Fungal strains, media and growth conditions

The wild-type strains of *F. fujikuroi* (FfWT) IMI58289 (Commonwealth Mycological Institute, Kew, UK) and *F. graminearum* (FgWT) PH-1 (FGSC 9075, NRRL 31084) were used as parental strains for deletion as well as overexpression experiments. For protoplasting, DNA isolation and western blot analyses, all strains were grown in darkness for three days on solid complete media (CM) (Pontecorvo et al., 1953) covered with cellophane sheets (Folia Bringmann) at 30 °C and 20 °C in the case of *F. fujikuroi* and *F. graminearum*, respectively. Fungal growth tests were performed on complete (CM, potato dextrose agar PDA) and minimal (synthetic ICI) media (Geissman et al., 1966) for 5 days under dark conditions at 30 °C and 20 °C for *F. fujikuroi* and *F. graminearum,* respectively. Additionally, fungal growth was monitored with a plate reader assay as described by Cánovas et al. (2017). Briefly, a 96-well plate containing 100 µL of desired solid medium (CM or ICI) was inoculated with 1,000 and 10,000 spores and incubated at 28 °C and 24 °C in the case of *F. fujikuroi* and *F. graminearum,* respectively. Absorbance was quantified at 595 nm every 60 min for 72 hrs in a synergy HT micro plate reader (Biotek). Medium inoculated with water only served as negative control, and was used to cancel background noises. The experiments were performed twice with technical triplicates for each strain and *Fusarium* spp. Fungal abiotic stress tests were performed on complete media supplemented with 1 M sorbitol and 1 M NaCl for osmotic stress; 5, 10 and 25 mM H_2O_2 and 0.25 and 0.5 mM menadione-sodium bisulfite (MSB) for oxidative stress (Ram and Klis, 2006). Both growth and stress plate assays were inoculated either with agar plugs (5 mm in diameter) or with 1,000 spores. To account for stress-independent growth deficiencies, growth on supplemented media was related to the growth on the non-supplemented media in the respective strains.

For *F. fujikuroi* SM analysis, the respective strains were pre-grown for 72 h in 300 mL Erlenmeyer flasks with 100 mL Darken medium (DVK) (Darken et al., 1959) on a rotary shaker under dark conditions at 180 rpm and 30 °C. A 500 µL aliquot of this culture was used for inoculation of synthetic ICI (Imperial Chemical Industries, Ltd, UK) medium (Geissman et al., 1966) with 6 mM or 60 mM glutamine as sole nitrogen source. Incubation was carried out for additional 7 days prior to chemical analyses. For *F. graminearum* SM analysis, the respective strains were grown on PDA plates for 14 days in darkness at 20 °C for two weeks as described by Giese et al. (2013). For conidia production, *F. fujikuroi* strains were grown for 7 days on solid V8 medium (20% v/v vegetable juice, Campbell Food, Puurs, Belgium) at 20 °C and 12 h/12 h light–dark cycles. In the case of *F. graminearum*, the strains were grown in 50 mL mung bean broth in 250 mL baffled flasks at 20 °C and 140 rpm for three days under dark conditions to induce conidiation (Bai and Shaner, 1996).

2.2. Plasmid construction

Plasmids for *F. fujikuroi* and *F .graminearum* deletion, complementation and overexpression strains were generated using yeast recombinational cloning (Colot et al., 2006; Schumacher, 2012). All primers used for polymerase chain reaction (PCR) were obtained from Sigma-Aldrich GmbH. For all generated constructs, the strategy and primers used in this study are displayed in Figure S2 A and Table S1. For deletion constructs, the upstream (5′) and downstream (3′) sequences of *FfKMT5* and *FgKMT5* were amplified from FfWT and FgWT genomic DNA, respectively. For this the following primer pairs were used: FfKmt5_5F and FfKmt5_5R for upstream, FfKmt5_3F and FfKmt5_3R for downstream regions in the case of *F. fujikuroi* and FgKmt5_5F and FgKmt5_5R for upstream and FgKmt5_3F and Fg_Kmt5_3R for downstream regions, respectively, in the case of *F. graminearum*. For both deletion constructs, hygromycin B was used for selection of positive transformants. The *hph* resistance cassette was amplified from pCSN44 (Staben et al., 1989) with the primer pair Hph_F and Hph_R. Complementation constructs were generated by amplifying the wild-type gene *FfKMT5/FgKMT5* from parental genomic DNA. For both fusaria, *FfKMT5/FgKMT5* are driven by the native promotor and amplified with the primer pairs FfKmt5_5F and FfKmt5_Cil_1R in the case of *F. fujikuroi* and FgKmt5_5F and FgKmt5_ORF_Tgluc_R in the case of *F. graminearum*. Both genes, *FfKMT5/FgKMT5*, were fused to the glucanase terminator of *Botrytis cinerea* (BcTgluc) followed by the geneticin (G418) resistance cassette, genR, amplified from p*Δfgkdm5/FgKDM5* (Bachleitner et al., 2019) using the primer pair Geni-F and Geni-Tgluc-R. Downstream sequences of *FfKMT5/FgKMT5* were amplified using the primer pairs FfKmt5_3F//FfKmt5_3R and FgKmt5_3F//FgKmt5_3R in the case of *F. fujikuroi* and *F. gramineraum*, respectively. Overexpression constructs were generated by amplifying the upstream (5′) sequence of *FfKMT5* and *FgKMT5* from

FfWT and FgWT genomic DNA, respectively, with the following primer pairs: FfKmt5_5F and YRC_OE_FfKmt5_hph_R in the case of *F. fujikuroi* and FgKmt5_5F and YRC_OE_FgKmt5_hph_R in the case of *F. graminearum*. The upstream sequence was followed by the *hph* resistance cassette and the *Aspergillus nidulans oliC* promotor, which was amplified from pOE::*KDM5* (Janevska et al., 2018b), with the primers TtrpC-hphR and pOliCR1 for both *F. fujikuroi* and *F. graminearum*. The constitutive *oliC* promoter was followed by the native *KMT5* gene and downstream (3′) sequence, amplified with the primers YRC_OE_pOliC_FfKmt5_F and FfKmt5_3R from FfWT gDNA in the case of *F. fujikuroi* and YRC_OE_pOliC_FgKmt5_F and FgKmt5_3R from FgWT gDNA in the case of *F. graminearum*, respectively. For yeast recombinational cloning, the *Saccharomyces cerevisiae* FY834 was transformed with the obtained fragments yielding p*Δffkmt5,* p*Δfgkmt5*, p*FfKMT5*^{Cis}, p*FgKMT5*^{Cis} (complementation in situ), pOE::*FfKMT5* and pOE::*FgKMT5* (overexpression). Correct assembly of the gained plasmids was verified by restriction digest and/or sequencing.

2.3. Fungal transformation

Transformation of *F. fujikuroi* IMI58289 was performed as described by Wiemann et al. (2013). For this, the deletion fragment was amplified from p*Δffkmt5* with the primer pair FfKmt5_5F and Ff_Kmt5_3R using a proof-reading polymerase (Q5-polymerase, New England Biolabs). For complementation, roughly 10 μg of p*FfKMT5*^{Cis} was digested with *Bgl*I prior to transformation. In the case of *F. graminearum*, the split marker approach was used (Goswami, 2012). For this, the deletion fragment was amplified with primer pairs FgKmt5_5F//Hph_split_F and FgKmt5_3R//Hph_split_R from p*Δfgkmt5,* whereas the complementation fragment was amplified with the primer pairs FgKmt5_5F//Geni_splitF and FgKmt5_3R//Geni_splitR from p*FgKMT5*^{Cis}. Transformed protoplasts were regenerated as described by Studt et al. (2017). Positive transformants were selected on regeneration media containing 100 ppm (parts per million) of either hygromycin or geneticin.

In the case of Δ*ffkmt5* and Δ*fgkmt5* mutants, homologous recombination events were verified with the primer pair dia_FfKMT5_F/dia_FgKMT5_F and pCSN44_trpC_T for the upstream part and dia_FfKMT5_R/dia_FgKMT5_R and pCSN44_trpC_P2 for the downstream part (Fig. S2 A/B). Absence of the native wild-type gene was verified with the primers dia_FfKMT5_WT_F//dia_FfKMT5_WT_R and dia_FgKmt5_WT_F//dia_FgKmt5_WT_R in the case of *F. fujikuroi* and *F. graminearum*, respectively (Fig. S2 B). Accordingly, homologous integration of the complementation constructs was verified using the primer pairs dia_FfKmt5_3′//pKS-Gengpd_P and dia _FgKmt5_R//pKS-Gengpd_P (downstream region) for *F. fujikuroi* and *F. graminearum,* respectively. Presence of the native wild-type gene i.e., *FfKMT5/FgKMT5*, was verified using the primers dia_FfKMT5_WT_F//dia_FfKMT5_WT_R (*FfKMT5*) and dia_FgKmt5_WT_F//dia_FgKmt5_WT_R (*FgKMT5*) (Fig. S3 A). Absence of *hph* was verified by the inability to grow on CM plates supplemented with hygromycin B (data not shown). In the case of OE::*FfKMT5* and OE::*FgKMT5*, homologous recombination events were verified with the primer pair dia_FfKMT5_F/dia_FgKMT5_F and pCSN44_trpC_T for the upstream part and dia_FfKMT5_R/dia_FgKMT5_R and pCSN44_trpC_P2 for the downstream part. Presence of the native wild-type gene, *FfKMT5/FgKMT5*, was verified using the primers FfKMT5_WT_F//FfKMT5_WT_R (*FfKMT5*) and FgKmt5_WT_F//FgKmt5_WT_R (*FgKMT5*) (Fig. S3 B). Altogether, Δ*ffkmt5*_T3, Δ*ffkmt5*_T4 and Δ*ffkmt5*_T7 as well as Δ*fgkmt5*_T11 and Δ*fgkmt5*_T14, were obtained for *F. fujikuroi* and *F. graminearum*, respectively. All mutants of *F. fujikuroi* and *F. graminearum* showed an identical phenotype. Hence, Δ*ffkmt5*_T3 and Δ*fgkmt5*_T11 were arbitrarily chosen for complementation approaches. This resulted in at least two independent mutants each for *F. fujikuroi* (Δ*ffkmt5/FfKMT5*^{Cis}_T1, T6, T9 and T14) and *F. graminearum* (Δ*fgkmt5/FgKMT5*^{Cis}_T9 and T12), that showed identical phenotypes and correct in situ integration of *FfKMT5* and *FgKMT5*, respectively (Fig. S3A). Overexpression of *FfKMT5* and *FgKMT5* was achieved by exchanging the native promoters with the constitutive strong *oliC* promoter from *A. nidulans*. At least three independent mutants overexpressing *FfKMT5* and *FgKMT5* were obtained for *F. fujikuroi* (OE::*FfKMT5*_T2, T3 and T11) and *F. graminearum* (OE::*FgKMT5*_T2, T10 and T11), respectively (Fig. S3B).

2.4. Standard molecular techniques

For DNA isolation, lyophilised mycelium was ground to a fine powder, re-suspended in extraction buffer and isolated as previously described (Cenis, 1992). Isolated genomic DNA was used for PCR amplification and Southern blot analysis. Deletion and complementation fragments were amplified with the proof-reading Q5 DNA Polymerase (New Englang Biolabs) and PCR reactions were set up according to the manufacturers' protocol. For diagnostic PCR, the GoTaq® Green Master Mix (Promega) was used and the PCR reactions were set up according to the users' manual. For Southern blot analyses, genomic DNA of FfWT/FgWT and the respective mutant strains i.e., Δ*ffkmt5/*Δ*fgkmt5* were digested with the enzyme *Xho*I. The digested DNA was separated on a 1% (w/v) agarose gel and subsequently transferred onto positively charged nylon membranes (Roche Diagnostics GmbH, Germany) by downward blotting. Probes were labelled with DIG-11-dUTP using the DIG-High Prime DNA Labelling and Detection Starter Kit II from Roche. In the case of *F. fukikuroi*, the downstream region, amplified with the primers FfKmt5_3F//FfKmt5_3R, was used for probing. In the case of *F. graminearum*, *hph* was used for probing and amplified with the primer pair Hph_F//Hph_R.

For expression analyses, RNA was extracted from lyophilised mycelium using the TRIzol Reagent (Thermo Fisher Scientific) according to the manufacturers' instruction. For cDNA synthesis 1 μg of total RNA was treated with DNaseI (Thermo Fisher Scientific) and subsequently reversely transcribed using the iScript™ cDNA Synthesis Kit (BioRad). RT-qPCR was performed with iQ SYBR Green Supermix (Bio-Rad, Munich, Germany) using an iCycler iQ Real-Time PCR System (Bio-Rad). To quantify the *KMT5* expression, the primers RT-qPCR_FfKmt5_F//R were used in the case of *F. fujikuroi* and RT-qPCR_FgKmt5_F//R in the case of *F. graminearum*, respectively (Table S1). The cDNA levels were related to the constitutively expressed reference genes actin (FFUJ_02611, FGSG_07335), β-tubulin (FFUJ_07385, FGSG_09530) and glyceraldehyde 3-phosphate dehydrogenase (FFUJ_13490, FGSG_06257). Used primers are listed in Table S1. Primer efficiencies in the RT-qPCR were kept between 90 and 110%. Relative expression levels were calculated using the ΔΔCt method (Pfaffl, 2001) and normalised to the respective wild type. Generated plasmids were extracted and purified from *E. coli* and *S. cerevisiae* with the GeneJET™ plasmid miniprep kit (Fermentas GmbH, St. Leon-Rot, Germany). For western blot analyses, mycelium from 3 days-old strains was ground to a fine powder and proteins were extracted as described (Studt et al., 2016). Depending on the antibody, 15–50 μg of proteins were used for SDS-Page and subsequent western blotting. The membrane was probed with 1:4,000 dilutions of anti-H3 C-Term (Active Motif, AM39451), anti-H4K20me3 (AM39671 and Abcam, ab9053), anti-H4K20me2 (AM35482) and anti-H4K20me1 (AM39728) primary antibodies and 1:10,000 dilutions of anti-mouse (Jackson, AB_10015289) HRP conjugated secondary antibody. Chemiluminescence was detected with Clarity™ ECL Western Substrate and ChemDoc™ XRS (Bio-Rad). Each western blot was performed at least two times for both fusaria.

2.5. Pathogenicity assays

F. fujikuroi infection assays were performed according to Wiemann et al. (2013). In detail, five germinated rice seedlings (*Oryza sativa* sp. japonica cv. Nipponbare) were inoculated with each strain. In the case of Δ*ffkmt5*, Δ*ffkmt5/FfKMT5*^{Cis} and OE::*FfKMT5* at least two independent mutants were used. After 14 days at 28 °C, 80% humidity and a 12 h/12 h light–dark cycle, rice plants were screened for typical bakanae disease

symptoms and pictures were taken. Non-infected rice seedlings served as a negative control (MOCK). For *F. graminearum* infections the highly susceptible (FHB) USU-Apogee full-dwarf hard red spring wheat (*Triticum aestivum* cv. USU-Apogee; Reg.no CV-840, PI592742) was used. Infection experiments were carried out in biological triplicates and are described elsewhere (Studt et al., 2017). Briefly, for each biological replicate, two spikelets of five individual wheat plants were infected with 1000 spores each. As a control, ears were inoculated with sterile water only (MOCK). After each treatment, the ears were covered in moistened plastic bags for the first 24 h to provide high humidity. Incubation conditions were set to 60% humidity, 20 °C for 16 h (day) and 18 °C for 8 h (night). The infection progress was monitored for up to 12 days.

In the case of *F. graminearum*, infection rate was determined as described elsewhere (Studt et al., 2017). Briefly, genomic DNA of infected plant material was extracted using the DNeasy® Plant Mini Kit (Qiagen). The infection rate was determined by qPCR quantification of the fungal DNA within the fungus/plant gDNA mixture. qPCR analysis

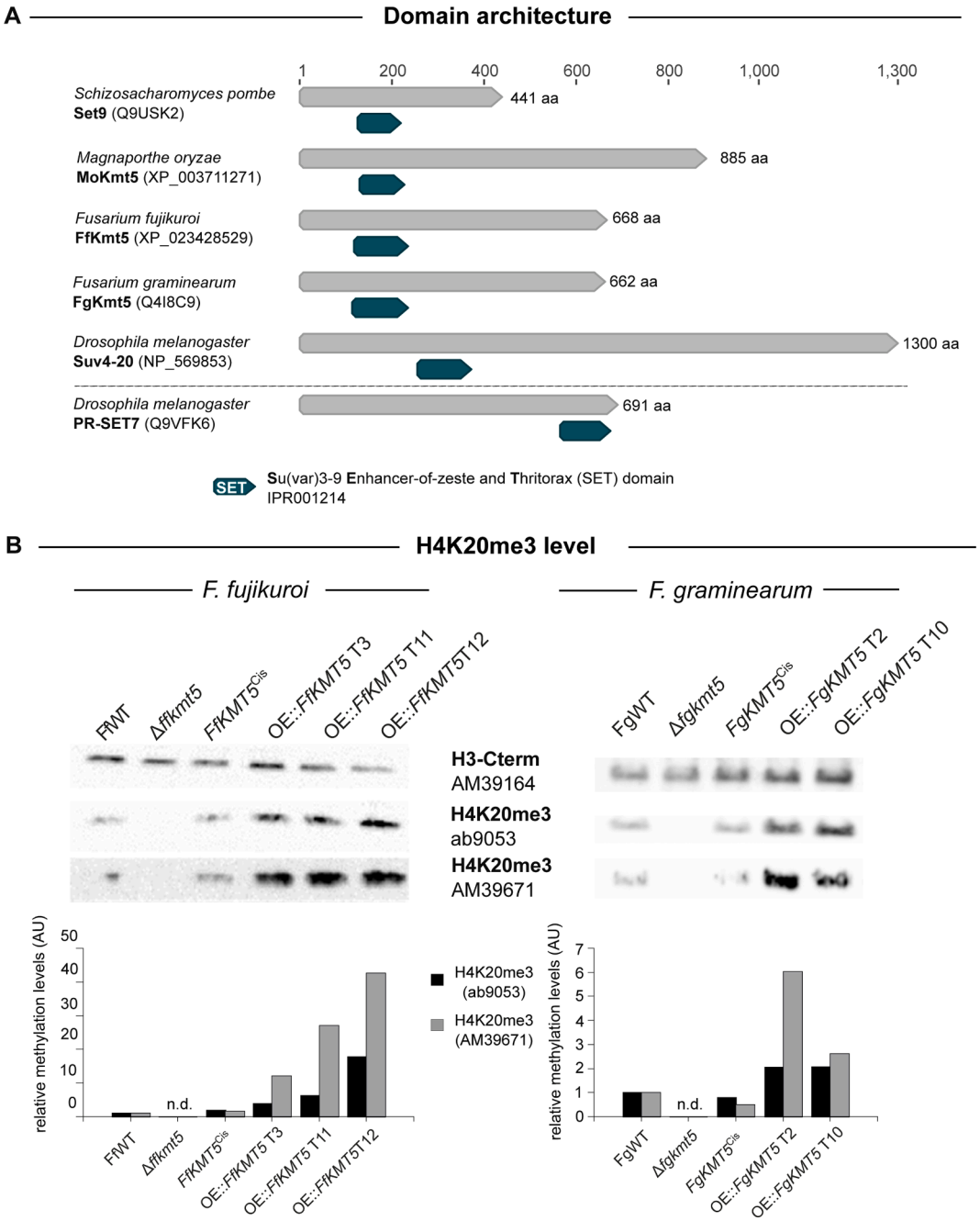

Fig. 1. FfKmt5 and FgKmt5 are the sole methyltransferases for H4K20 tri-methylation in *F. fujikuroi* and *F. graminearum*. **(A)** The conserved Kmt5 domain structure is shown for *Schizosaccharomyces pombe*, *Magnaporthe oryzae*, *F. fujikuroi* and *F. graminearum* as well as for the higher eukaryote *D. melanogaster*. In contrast to filamentous fungi and yeast, *D. melanogaster* harbours additionally to the H4K20 mono-methyltransferase PR-SET7, another enzyme i.e., Suv4-20 that is responsible for di- and tri-methylation of H4K20. Domain analysis was performed with protein sequences obtained from NCBI (accession number for each protein is shown in figure) in Geneious Prime 2020.2.4. **(B)** For western blot analysis, indicated strains were grown on complete media (CM) plates. Whole protein extracts were isolated from lyophilized mycelia and roughly 15 µg of proteins were used for western analysis. H3 C-Term and H4K20me3 antibodies were used for detection. Experiments were done in triplicates showing the same result. Hence, only one experiment is depicted here. For quantification a densitometric analysis was performed, and the respective wild-type strain was arbitrarily set to 1; AU, artificial units.

was performed using the iQTM SYBR Green Supermix (Bio-Rad) and primers Pks12_ORF_fwd//Pks12_ORF_rev for quantification of *F. graminearum* gDNA as well as primers ITS1P//ITS4 for quantification of plant gDNA.

2.6. Chemical analysis

Analysis of all here studied *F. fujikuroi* SMs was accomplished using the culture fluids of 7 days-old cultures that were directly used for analyses without further preparation. *F. graminearum* strains were grown for two weeks on potato dextrose agar (PDA) plates and subsequently extracted using MeOH/CH_2Cl_2/EtOAc (1/2/3, v/v), evaporated and resuspended in methanol/H_2O (1/1, v/v) as previously described (Sondergaard et al., 2016). For quantification of GA_3 and DON levels of infected rice and wheat plants, freeze-dried and lyophilised plant material was pooled prior extraction (Studt et al., 2017). The samples were run on a QTrap 5500 LC–MS/MS System (Applied Biosystems, Foster City, CA, USA) equipped with a TurboIonSpray electrospray ionisation (ESI) source and a 1290 Series HPLC System (Agilent, Waldbronn, Germany). Chromatographic separation was done at 25 °C using a Gemini C18 150 × 4.6 mm i.d., 5 µm particle size, equipped with a C18 3 × 4 mm i.d. security guard cartridge (Phenomenex, Torrance, CA, USA). The chromatographic method and chromatographic and mass spectrometric parameters are described elsewhere (Sulyok et al., 2020).

3. Results

3.1. The histone methyltransferases FfKmt5 and FgKmt5 are solely responsible for mono-, di- and trimethylation of H4K20 in F. fujikuroi and F. graminearum, respectively

The KMT5 homologues in *F. fujikuroi* and *F. graminearum*, FfKmt5 and FgKmt5, respectively, were identified by determining the orthologues using QuartetS (Yu et al., 2011). The predicted proteins, FFUJ_03480 (FfKmt5) and FGSG_06529 (FgKmt5) in the case of *F. fujikuroi* and *F. graminearum*, respectively, are the orthologues of the H4K20-specific methyltransferase MoKmt5 (MGG_07393) in *Magnaporthe oryzae* (Pham et al., 2015). Pairwise sequence alignment using LALIGN (Huang and Miller, 1991) showed 82.1% sequence identity (E-value 6.9e-172) of Kmt5 on protein level between the two fusaria, but only 48.5% (*F. fujikuroi*) and 48.2% (*F. graminearum*) sequence identity to *M. oryzae*. For illustration, all three sequences were aligned by T-coffee (Notredame et al., 2000) and formatted with BOXSHADE program (Fig. S1).

Similar to the homologue Set9 in *Schizosaccharomyces pombe* and MoKmt5 in *M. oryzae*, both FfKmt5 and FgKmt5 contain a catalytically active SET domain (Fig. 1 A). Contrary to vertebrates, fungi harbour only one KMT5 homologue, probably responsible for all three methylation degrees (Sanders et al., 2004) while higher eukaryotes harbour a H4K20 mono-methyltransferase (PR-Set7/Set8) (Fang et al., 2002; Nishioka et al., 2002) as well as one (*Drosophila melanogaster*) or more (vertebrates) di-and tri-methyltransferases (Suv4-20 enzymes) (Sakaguchi et al., 2008; Schotta et al., 2004). Notably, sequence analysis revealed that Set9 is more similar to Suv4-20 enzymes than to PR-SET7 (Greeson et al., 2008) (Fig. 1 A).

To study the functions of FfKmt5 and FgKmt5, the respective *KMT5* open reading frames were deleted in the wild-type strains i.e., IMI58289 (*F. fujikuroi*) and PH-1 (*F. graminearum*), hereafter referred to as FfWT and FgWT, respectively (Fig. S2). Correct integration of the resistance cassettes was subsequently verified by diagnostic PCR and Southern blot analysis (Fig. S2 B/C). To test whether FfKmt5 and FgKmt5 are involved in writing H4K20 methylation in *F. fujikuroi* and *F. graminearum*, respectively, wild-type and the respective deletion strains were applied for western blotting using specific antibodies. All three methylation degrees of H4K20 were completely abolished in both Δ*ffkmt5* and Δ*fgkmt5* mutants (Fig. S5). To verify the obtained results, we next approached complementation of Δ*ffkmt5* and Δ*fgkmt5* as well as overexpression of *KMT5* in both fusaria (Fig. S3). Notably, *KMT5* transcript levels were restored to wild-type levels in the complemented strains, Δ*ffkmt5/FfKMT5*^{Cis} and Δ*fgkmt5/FgKMT5*^{Cis} (hereafter referred to as *FfKMT5*^{Cis} and *FgKMT5*^{Cis}), and increased to about 10-fold in the overexpression strains, OE::*FfKMT5* and OE::*FgKMT5* when compared to the respective wild types (Fig. S4). Consistent with the expression data, H4K20me3 signals were wild type-like in the complemented strains and significantly increased in the OE::*FfKMT5* and OE::*FgKMT5* strains (Fig. 1B). Thus, FfKmt5 and FgKmt5 are solely responsible for writing mono-, di- and trimethylation of H4K20 in *F. fujikuroi* and *F. graminearum*, respectively.

3.2. FgKmt5 but not FfKmt5 significantly affects fungal colony growth

To gain insights into the importance of H4K20me for fungal growth, we monitored radial hyphal growth by performing plate assays using complete (CM and potato dextrose agar, PDA) as well as minimal (synthetic ICI medium supplemented with 6 mM glutamine as sole nitrogen source) media. To cover not only differences in fungal growth but also possible defects in germination, plates were inoculated with the relevant strains using agar plugs as well as conidia. No or only minor differences were detectable between Δ*ffkmt5* and OE::*FfKMT5* compared to FfWT, respectively, on both complete and minimal medium (Fig. 2A/B). In contrast to this, lack of FgKmt5 in *F. graminearum* resulted in significantly reduced radial growth on ICI and CM but not on PDA (Fig. 2A/B). Notably, the growth defect of Δ*fgkmt5* was more obvious when inoculated with agar plugs, while conidia-inoculated plates converged to a wild type-like phenotype. Radial hyphal growth was reduced to 64% and 58% on ICI and CM, respectively, compared to FgWT when inoculated with agar plugs. However, when conidia were used as inoculum instead of agar plugs, the previously detected growth defect for Δ*fgkmt5* resembled a wild type-like phenotype. Here, only slight differences of growth were detected i.e., 75% and 90% on ICI and CM, respectively (Fig. 2A/B). As expected, complementation of Δ*fgkmt5* i.e., *FgKMT5*^{Cis}, restored the wild-type phenotype with regard to hyphal growth on all tested media. Notably, radial hyphal growth was also affected in OE::*FgKMT5* strains although to a lesser extent. Here, radial hyphal growth was significantly reduced to 75% and 78% on ICI and PDA, respectively, compared to FgWT when agar plugs were used as inoculum. When conidia were used as inoculum instead of agar plugs, the previous detected growth defect for OE::*FgKMT5* was still detectable on ICI (78% of FgWT) but not on PDA plates (Fig. 2A/B).

Radial hyphal extension (linear growth) does not necessarily reflect biomass accumulation. To evaluate whether biomass formation is indeed reduced in Δ*fgkmt5* strains, we next performed plate reader assays with the *KMT5* deletion strains compared to both wild types. For this, absorbance (OD595 nm) was measured of fungal colonies growing on top of solid CM as well as on minimal medium (synthetic ICI with 6 mM glutamine as sole nitrogen source) as described (Cánovas et al., 2017). As expected, when grown on solid complete or minimal media, no differences in exponential growth were observed for Δ*ffkmt5* compared to FfWT (Fig. S6). In agreement with the plate assay, biomass formation was reduced to 78% on minimal medium (synthetic ICI) in Δ*fgkmt5* compared to FgWT, while no differences were observed when grown on solid CM (Fig. S6).

Next, we quantified spore formation in the two fusaria. For this, the respective strains were cultivated on solid V8 medium or in mung bean broth in the case of *F. fujikuroi* and *F. graminearum*, respectively. No significant differences in spore quantity were detectable for the *KMT5* deletion or overexpression strains when compared to the respective wild types in both fusaria (Fig. S7). This suggests that Kmt5 is not involved in asexual development in these fungi. Thus, deletion of *KMT5* impacts hyphal growth in *F. graminearum* but not in *F. fujikuroi*, while asexual development remains unaltered.

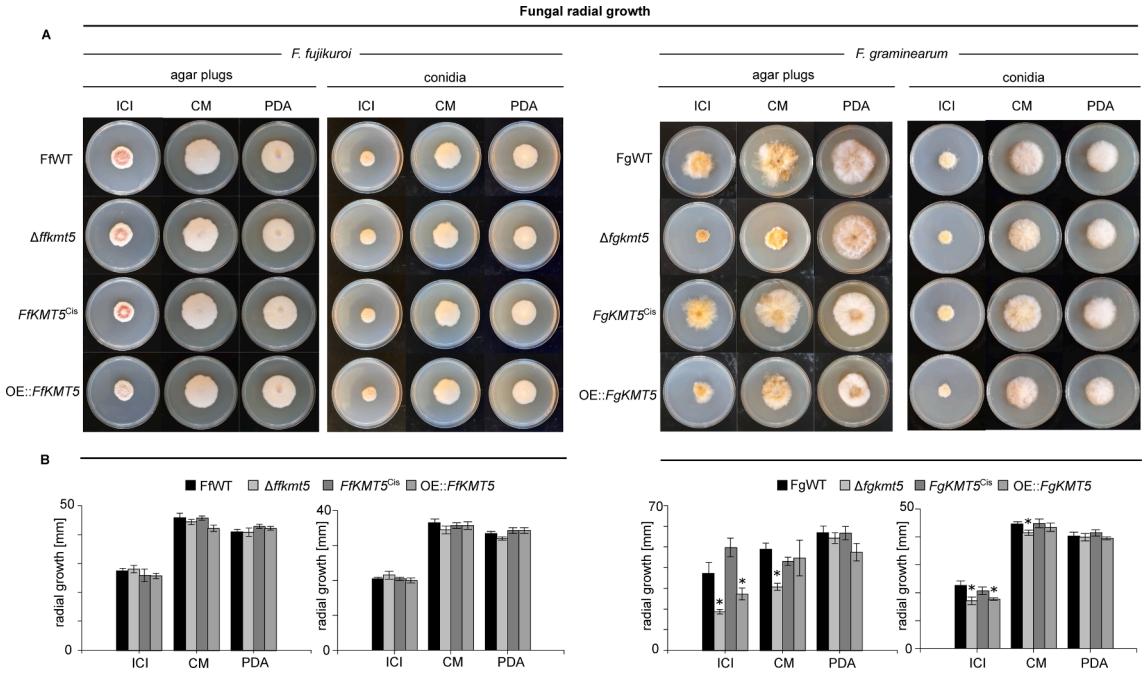

Fig. 2. Loss of Kmt5 results in impaired radial hyphal growth in *F. graminearum* but not in *F. fujikuroi*. (A) For growth analysis indicated strains were inoculated either with agar plugs or conidia on minimal media (ICI supplemented with 6 mM glutamine) and complete media (CM, PDA) and incubated at 30 °C and 20 °C for *F. fujikuroi* and *F. graminearum*, respectively. After five days post inoculation pictures were taken. **(B)** Experiments were performed in technical and biological triplicates. All experiments showed the same result, thus only one is depicted here. Mean values and standard deviations are shown, *p < 0.05.

3.3. Both FfKmt5 and FgKmt5 are crucial for wild type-like secondary metabolism

Fusaria are known for their ability to produce a broad spectrum of SMs. Thus, we were interested to see if loss or overexpression of *KMT5* affects SM biosynthesis in *F. fujikuroi* and *F. graminearum*. Both species produce sets of common but also species-specific SMs (Sieber et al., 2014; Wiemann et al., 2013). To analyse changes in secondary metabolism, *F. fujikuroi* strains were cultivated for seven days in synthetic ICI under nitrogen limited (6 mM glutamine) or nitrogen surplus (60 mM glutamine) conditions (Studt et al., 2017). *F. graminearum* strains were grown for two weeks on PDA plates prior to SM extraction as described by Giese et al. (2013). Overall, loss but also overexpression of *KMT5* had distinct effects on some of the analysed SMs in both species. In detail, we observed small deviations in gibberellin (GA_3) biosynthesis in *F. fujikuroi*. Here, overexpression but not deletion of *FfKMT5* reduced GA_3 levels to 67% of FfWT. It is noteworthy, that although Δ*ffkmt5* showed lower GA_3 levels, this trend was not significant. Similarly, gibepyrone D levels remained unaltered by loss or overexpression of *FfKMT5*. In contrast to GA_3 and gibeyrone D levels, fusarin levels were strongly affected upon loss of FfKmt5 and increased to 585% when compared to FfWT. However, fusarin levels remained unaffected in OE::*FfKMT5* strains. Further, bikaverin biosynthesis was significantly reduced to about 25% of FfWT in OE::*FfKMT5* only (Fig. 3A). In *F. graminearum*, OE::*FgKMT5* significantly increased DON levels to 229% of FgWT level, while DON biosynthesis remained wild type-like in Δ*fgkmt5*. Contrary to *F. fujikuroi*, fusarin but also zearalenone levels were decreased to 43% and 4% compared to FgWT in Δ*fgkmt5*, respectively. Biosynthesis of both compounds was not deviating from the wild type in OE::*FgKMT5* strains. Aurofusarin biosynthesis remained unaffected by both, Δ*fgkmt5* and OE::*FgKMT5* (Fig. 3B). As expected, the complementation strains i.e., *FfKMT5*^{Cis} and *FgKMT5*^{Cis}, phenocopied the respective wild-type strains with regards to SM levels in both fusaria. Thus, altered *KMT5* expression levels affected some SMs in both fusaria, with the most drastic phenotypes in fusarin biosynthesis in Δ*ffkmt5* in *F. fujikuroi* and zearalenone biosynthesis in Δ*fgkmt5* in *F. graminearum*. Notably, deletion and respective overexpression strains did not show opposing phenotypes in any of the analysed *Fusarium* spp.

3.4. FgKmt5 but not FfKmt5 is required for wild type-like symptom development during pathogenic interaction

As overexpression of *KMT5* affected biosynthesis of the virulence factors GA_3 and DON in axenic cultures in both fusaria, the role of Kmt5 in virulence and pathogenic development was investigated on rice (*F. fujikuroi*) and wheat heads (*F. graminearum*). In the case of *F. fujikuroi*, Δ*ffkmt5, FfKMT5*^{Cis} and OE::*FfKMT5* caused typical bakanae symptoms (shoot elongation and chlorosis) on rice seedlings, and internode elongation did not deviate from FfWT-infected rice seedlings (Fig. 4 A/B). The biosynthesis of GA_3 is directly associated with the yellowish chlorotic leaves and hyper-elongated internodes of infected rice seedlings (Bömke and Tudzynski, 2009). Consistent with the occurrence of bakanae symptoms, GA_3 levels quantified *in planta* were not affected in Δ*ffkmt5* or OE::*FfKMT5* when compared to FfWT (Fig. 4 C). The same is true for *F. graminearum*: Δ*fgkmt5* or OE::*FgKMT5* DON levels quantified *in planta* showed no deviations from both the FgWT and FgKMT5^{Cis} (Fig. 4C), which contradicts axenic cultivations in the case of OE::*FgKMT5* (Fig. 3B). Yet, strains lacking FgKmt5 were hypovirulent on wheat (Fig. 4 A/B). While FgWT, *FgKMT5*^{Cis} and OE::*FgKMT5* were able to infect about 8 spikelets over a time period of 10 days, Δ*fgkmt5* was not able to infect more than 6 spikelets. Since experiments using biological systems may cause wide standard deviations, the experiment was carried out in three biological replicates. In all three experiments, the same trend was observed. In line with the hypovirulence, lower infection rates were determined for Δ*fgkmt5* i.e., 84% of FgWT, whereas complemented and overexpressed strains reached 90.5% and 95% (Fig. S8). Thus, Δ*fgkmt5* is hypovirulent when compared to FgWT and *FgKMT5*^{Cis}. Whether this phenotype is truly virulence-related or can be attributed to an impaired growth of Δ*fgkmt5* strains remains elusive at this point. To sum up, Kmt5 is not required for wild type-like virulence in *F. fujikuroi*, which is in line with previous observations in *M. oryzae* (Pham et al., 2015), but impacts virulence in *F. graminearum*.

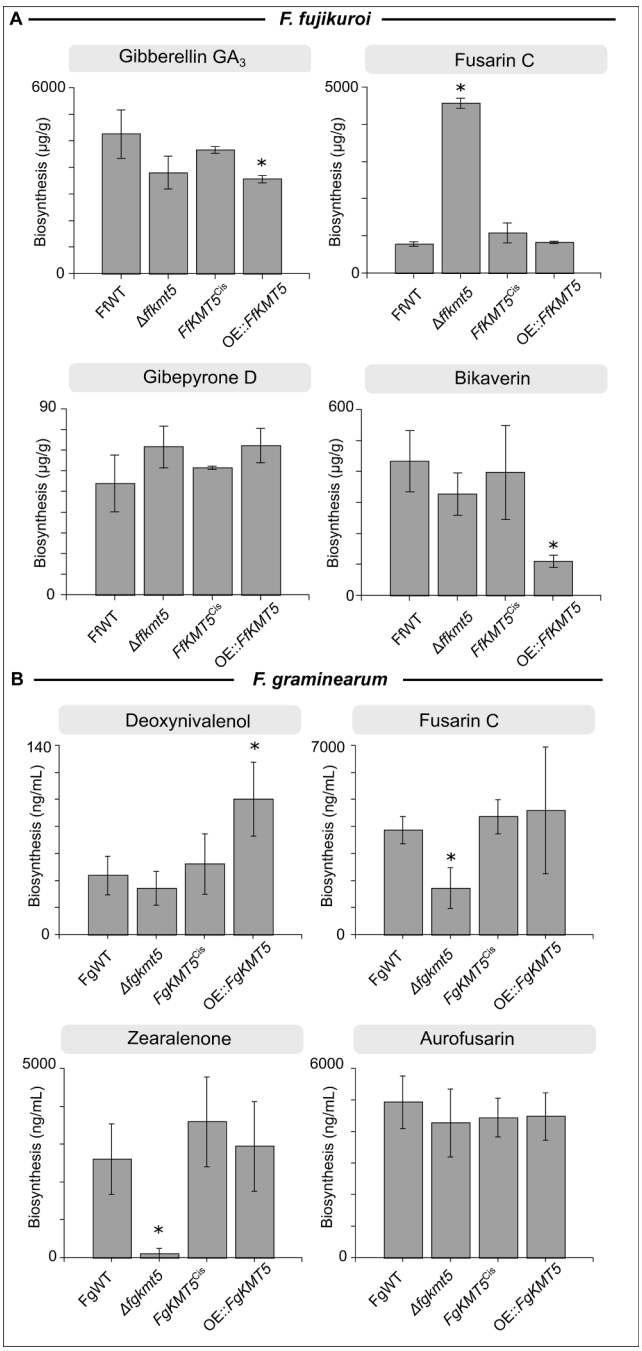

Fig. 3. Loss of Kmt5 affects secondary metabolism in both fusaria. (A) For secondary metabolite analysis in *F. fujikuroi*, strains were cultured in liquid ICI (supplemented with 6 mM glutamine for giberellin, bikaverin and gibepyron, and 60 mM glutamine for fusarin C biosynthesis) for seven days at 30 °C and 180 rpm. **(B)** *F. graminearum* strains were grown on PDA plates for two weeks and subsequently extracted as described in Materials and Methods. Experiments were done in triplicates. Mean values and standard deviations are shown; *p < 0.05.

3.5. Lack of Kmt5 results in mutants that are more tolerant to menadione sodium bisulfite-induced stress

Oxidative stress response protects organisms from harmful effects of reactive oxygen species (ROS), which can damage DNA, cellular components and cause disturbance of the cellular homeostasis (Angelova et al., 2005; Morano et al., 2012). H4K20me2 has been shown to be an important mark for genomic maintenance and DNA damage response (Paquin and Howlett, 2018). Thus, we next analysed whether FfKmt5 and/or FgKmt5 plays a role in the cellular redox balance during the adaptation to environmental changes. For this, radial hyphal growth was investigated in the presence of two widely used agents to study oxidant stress i.e., hydrogen peroxide (H_2O_2) and menadione sodium bisulfite (MSB – a water-soluble derivate of menadione/vitamin K3). MSB has the ability to easily cross membranes and catalyses reactions, which release superoxide, hydrogen peroxide and hydroxyl radicals (Lehmann et al., 2012; Rasheed et al., 2018). *KMT5* deletion, complementation and overexpression strains of both fusaria were grown on solid CM supplemented with either H_2O_2 or MSB. As differences of previous detected growth defects of Δ*fgkmt5* were linked to the type of inoculation, we inoculated the relevant strains either with agar plugs or conidia. Furthermore, growth on media supplemented with stressors was normalised with normal growth on CM plates of the respective strain. After five days of fungal growth no deviations were detected for all strains on CM supplemented with H_2O_2 when agar plugs were used (Fig. S9). In the case of conidia-inoculated plates, Δ*ffkmt5* did not deviate from the wild type. However, *FfKMT5*^{Cis} and OE::*FfKMT5* showed retarded growth i.e., 80% and 73%, respectively, compared to FfWT on 5 mM H_2O_2 plates. Similar to *F. fujikuroi*, no growth deviations were observed for Δ*fgkmt5* but slightly increased growth was detected for *FgKMT5*^{Cis} and OE::*FgKMT5* i.e., 116% and 113% when compared to FgWT on 0.5 mM H_2O_2 and 115% and 107% on 1 mM H_2O_2 plates, respectively (Fig. S9). As the complementation strains are not able to complement the wild-type phenotype in both fusaria, these data need to be evaluated carefully. It is noteworthy, that conidia of *F. graminearum* were not able to germinate on CM plates supplemented with 2.5 mM H_2O_2 and higher. Thus, lower concentrations (0.5 and 1 mM H_2O_2) were chosen (Fig. S9).

While H_2O_2 treatment did not have a compelling impact on the growth of the strains, a different scenario was shown when MSB was used as a stressor. Here, both Δ*ffkmt5* and Δ*fgkmt5* showed significantly increased growth in presence of MSB compared to the respective wild-type strains in the case of both inoculation methods (Fig. 5). In the case of *F. fujikuroi*, growth of Δ*ffkmt5* was increased to 205% of wild-type level on 0.5 mM MSB when inoculated with agar plugs, and 124% of wild-type level on 0.25 mM MSB plates when inoculated with conidia, respectively. Noteworthy, conidia were not able to germinate on 0.5 mM MSB plates. Thus, lower concentrations (0.1 and 0.25 mM MSB) were used. The complemented strain i.e., *FfKMT5*^{Cis}, restored the wild type-growth phenotype in *F. fujikuroi*. Conversely, overexpression of *FfKMT5* resulted in strains that are more susceptible towards MSB as radial growth rates were reduced to 60% on agar plug-inoculated MSB (0.5 mM) and 76% on conidia-inoculated MSB (0.25 mM) plates (Fig. 5A/B). In the case of *F. graminearum*, growth of Δ*fgkmt5* was increased to 124% of wild-type level on agar plug-inoculated plates supplemented with 0.5 mM MSB, and to 164% of wild-type level on conidia-inoculated plates supplemented with 0.25 mM MSB. The *FgKMT5*^{Cis} strain restored the wild-type phenotype. In contrast to *F. fujikuroi*, OE::*FgKMT5* showed no consistent susceptibility towards MSB. Here, a reduced growth rate was only observed for conidia-inoculated plates supplemented with 0.1 mM MSB (Fig. 5A/B). To sum up, loss of Kmt5 resulted in elevated oxidative stress tolerance when treated with MSB in both fusaria. However, overexpression of *KMT5* reversed the phenotype only in *F. fujikuroi* but not in *F. graminearum*.

3.6. Lack of Kmt5 results in mutants that are more tolerant to osmotic stress

Next, we analysed the adaptation ability to osmotic stress by growth on solid CM supplemented with high salt (NaCl) and sugar (sorbitol) concentrations. For this, the relevant strains were inoculated with either agar plugs or conidia together with the respective wild-type strains on solid CM supplemented with either 1 M NaCl or 1 M sorbitol. In both fusaria, lack of Kmt5 resulted in a significantly increased tolerance

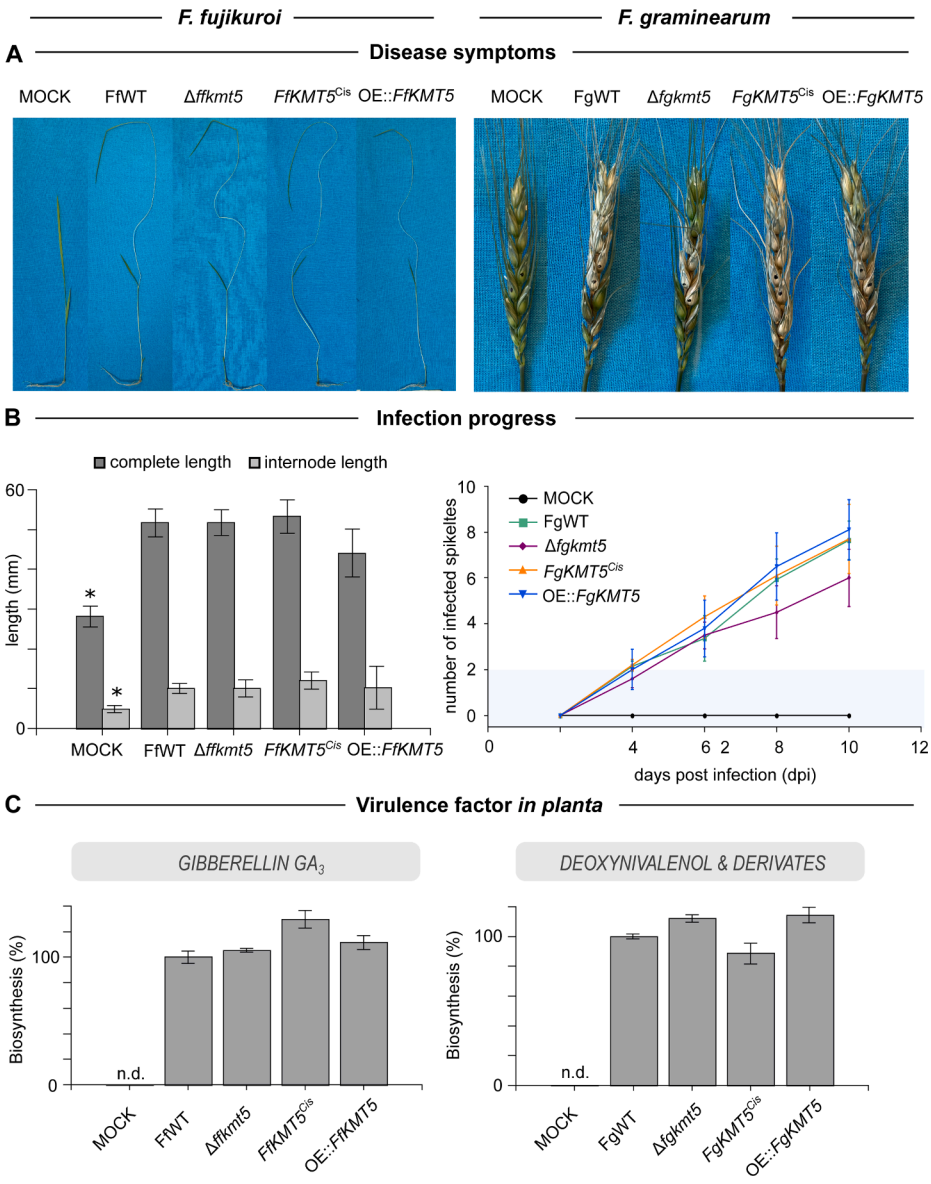

Fig. 4. Loss of FgKmt5 results in attenuated virulence on APOGEE wheat. (A) For pathogenicity analysis rice seedlings and wheat heads were infected with indicated *F. fujikuroi* and *F. graminearum* strains as described in Material and Methods. Pictures of rice plantlets and wheat heads were taken post-harvest. **(B)** Disease development was determined by measuring internode and complete length of rice plantlets in the case of *F. fujikuroi* infections. Wheat heads infected with *F. graminearum* strains were monitored over 10 days by measuring infected spikelets. Experiments were done in biological triplicates, resulting in the same trend, thus only one experiment is depicted here. **(C)** For in planta GA_3 and DON quantification, five plants were infected with indicated *F. fujikuroi* and *F. graminearum* strains. Plants were pooled and the total amount of GA_3 and DON was extracted and quantified by HPLC–MS/MS. Experiments were performed in biological triplicates; n.d., not detectable.

towards NaCl and sorbitol compared to the respective wild-type strains. In *F. fujikuroi*, radial growth of Δ*ffkmt5* was increased to 118% in presence of 1 M NaCl and 112% in presence of 1 M sorbitol when agar plugs were used as inoculum. (Fig. 6). Similarly, increased osmotic stress tolerance was detected for conidia-inoculated plates. Here, radial growth of Δ*ffkmt5* increased to 117% in presence of both, 1 M NaCl and 1 M sorbitol. The *FfKMT5*^{Cis} strain restored the wild-type phenotypes for both inoculation methods. Conversely to Δ*ffkmt5,* growth of OE::*FfKMT5* was slightly reduced on CM supplemented with 1 M sorbitol to 86% compared to FfWT, while no significant deviation was detected on NaCl supplemented plates (Fig. 6 A/B). A similar scenario was observed for *F. graminearum*. Here, growth of Δ*fgkmt5* was increased to 200% in presence of 1 M NaCl and 110% in presence of 1 M sorbitol when agar plugs were used as inoculum. Similarly, conidia-inoculated plates showed 110% and 116% increased growth for Δ*fgkmt5* in presence of 1 M NaCl and sorbitol, respectively. The complemented *FgKMT5*^{Cis} strain restored only partially the wild-type phenotypes, as *FgKMT5*^{Cis} was deviating from the respective wild type on agar-plug inoculated 1 M NaCl plates. In most cases, OE::*FgKmt5* was not deviating from FgWT, except on agar plug-inoculated 1 M NaCl plates. Here, growth was similarly to *FgKmt5*^{Cis} increased to 145% (Fig. 6A/B). Thus, loss of Kmt5 resulted in an increased tolerance towards osmotic stress in both fusaria, while only overexpression of *FfKMT5* reversed the phenotype on sorbitol-supplemented plates.

4. Discussion

Methylation of H4K20 is a well-studied histone PTM in animal cells and the fission yeast *Schizosaccharomyces pombe*. However, very little knowledge exists about its function in filamentous ascomycetes. In this study, we have identified and characterised the H4K20-specific methyltransferase Kmt5 in the two plant pathogenic fungi, *F. fujikuroi* and *F. graminearum*, by a reverse genetics approach.

4.1. Imbalanced H4K20me levels affect radial growth in F. graminearum but not in F. fujikuroi

While lack of *FfKMT5* did not affect morphology and growth in *F. fujikuroi*, we observed retarded growth for both the *FgKMT5* deletion and overexpression mutants on complete medium (agar plugs) and minimal media (ICI; conidia and agar plugs). This suggests that wild-type H4K20me levels are crucial for normal growth in *F. graminearum*.

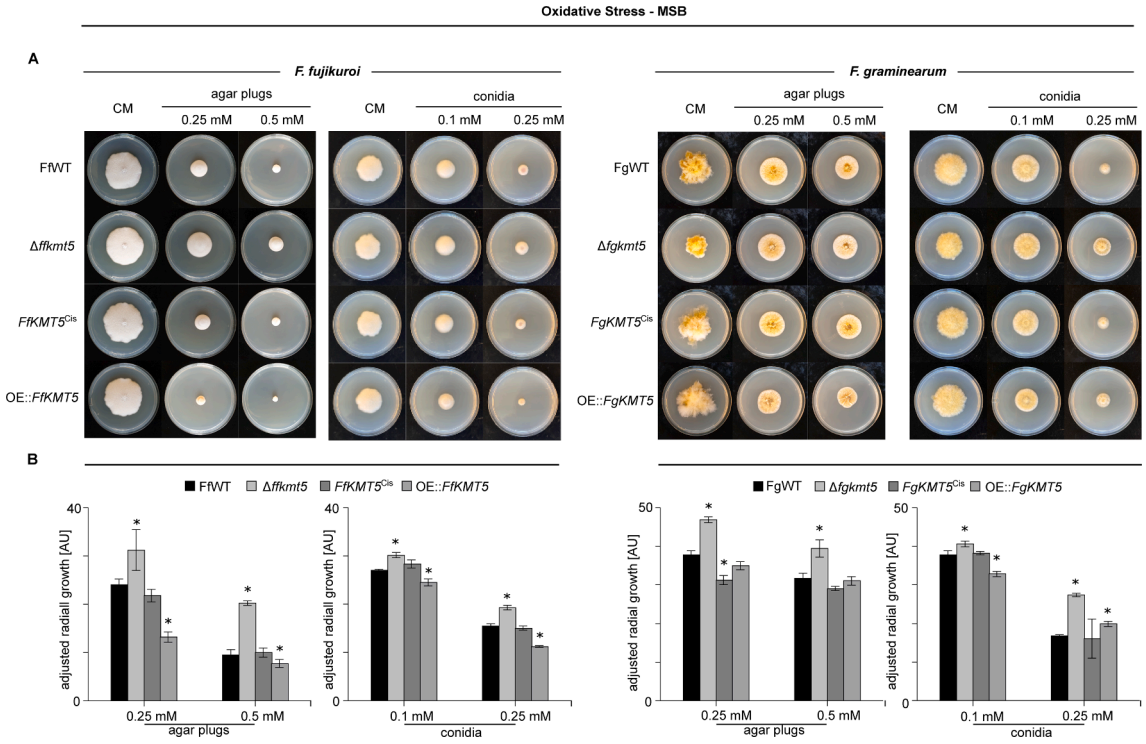

Fig. 5. Lack of Kmt5 results in strains more tolerant to menadione sodium bisulfite stress in *F. fujikuroi* and *F. graminearum*. (A) For oxidative stress analysis indicated strains were grown on complete media supplemented with 0.25 and 0.5 mM MSB when agar plugs were used as inoculum and 0.1 and 0.25 mM MSB when conidia were used for inoculation. Plates were cultivated at 30 °C and 20 °C in the case of *F. fujikuroi* and *F. graminearum* respectively. Pictures were taken after five days post inoculation. **(B)** Growth on media supplemented with stressors was adjusted to normal growth on CM plates of the respective strain. Experiments were performed in technical and biological triplicates. All experiments showed the same result, thus only one is depicted here. Mean values and standard deviations are shown; *p < 0.05, AU; artificial units.

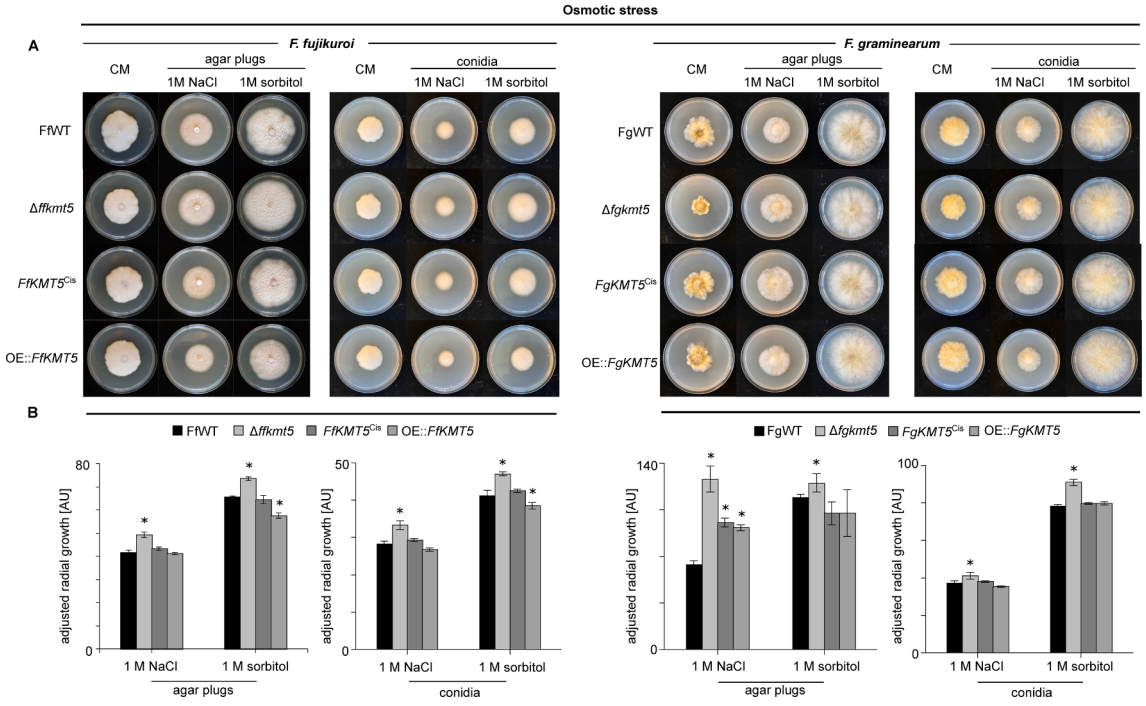

Fig. 6. Lack of Kmt5 results in strains more tolerant to osmotic stress in *F. fujikuroi* and *F. graminearum*. (A) For osmotic stress analysis indicated strains were grown on complete media supplemented with 1 M NaCl and 1 M sorbitol. Plates were inoculated either with agar plugs or conidia and incubated at 30 °C and 20 °C under dark conditions in the case of *F. fujikuroi* and *F. graminearum* respectively. Pictures were taken after five days post inoculation. **(B)** Growth on media supplemented with stressors was adjusted with normal growth on CM plates of the respective strain. Experiments were performed in technical and biological triplicates. All experiments showed the same result, thus only one is depicted here. Mean values and standard deviations are shown; *p < 0.05., AU; artificial units.

Impaired growth was also reported for the rice blast fungus *M. oryzae*. Here, a lower growth rate was reported for Δ*mokmt5* compared to the wild-type strain on complete media (Pham et al., 2015). Loss of the Kmt5 orthologue Set9 was shown to be nonessential for cell viability in *S. pombe* but significantly affects DNA damage response (Sanders et al., 2004). In *D. melanogaster* but also in mammalian cells, loss of PR-Set7 disturbs the normal cell cycle progression and leads to accumulated DNA damage (Jørgensen et al., 2007; Li et al., 2016; Oda et al., 2009; Tardat et al., 2007). If imbalanced H4K20 methylation levels result in defective cell cycle progression and thus lead to growth impairment in *F. graminearum* remains unanswered at this moment. Similarly, why imbalanced H4K20 methylation levels only affect growth in *F. graminearum* but not in *F. fujikuroi* is elusive at this point. Characterisation of Kmt5 homologues in diverse fungal species will shed further light onto these species-specific differences.

4.2. Kmt5 affects SM biosynthesis in both fusaria

Deletion as well as overexpression of *KMT5* led to significant changes in SM biosynthesis in both *Fusarium* spp. In detail, GA_3 and bikaverin levels were significantly decreased by overexpression of *FfKMT5* whereas fusarin levels were highly increased upon loss of FfKmt5 in *F. fujikuroi*. Thus, elevated H4K20me3 levels led presumably to a repression of these compounds and support the hypothesis of H4K20me3 as a silencing mark. However, a different scenario was shown for *F. graminearum*. Here, overexpression of *FgKMT5* resulted in significantly increased DON levels, while loss of FgKmt5 significantly reduced and almost abolished biosynthesis of fusarins and zearalenone, respectively. This suggests activating functions of FgKmt5 for these SMs. As *KMT5* deletion and overexpression strains did not show opposing phenotypes in any of the analysed *Fusarium* spp., we assume that alterations in SM biosynthesis are rather inflicted via secondary effects than by directly targeting SM biosynthetic genes. Notably, Xu and Kidder (2018) showed that H4K20me3 co-localises with H3K4me3 and H3K36me3 at transcriptionally dynamic regions in embryonic stem cells. Generally, H3K4me3 and H3K36me3 are considered hallmarks of active euchromatin (Black et al., 2012; Rivera et al., 2014), and many studies have highlighted their importance in SM regulation in filamentous fungi. For example, loss of Set1, the catalytic subunit of COMPASS, involved in H3K4 methylation reduced the expression of genes involved in DON and fumonisin biosynthesis in *F. graminearum* and *F. verticilloides*, respectively (Gu et al., 2017b; Liu et al., 2015). Similar results were obtained in *Aspergillus flavus*. Here, loss of Set1 led to abolished aflatoxin B1 biosynthesis (Liu et al., 2020). Notably, H3K4me3 was also shown to negatively impact SM biosynthesis. For example loss of another COMPASS component (CclA) resulted in increased emodin and monodictyphenone levels in *A. nidulans* (Bachleitner et al., 2019; Bok et al., 2009). Similarly, in *Aspergillus fumigatus* and *Aspergillus oryzae*, loss of *cclA* increased the biosynthesis of gliotoxin and astellolides, respectively (Palmer et al., 2013; Shinohara et al., 2016). Furthermore, biosynthesis of five novel terpenoid compounds was induced in Δ*cclA* mutants in *Colletotrichum higginsianum* (Dallery et al., 2019), and the subtelomeric gene clusters involved in ergotalkaloid (EAS) and lolitrem (LTM) biosynthesis were upregulated in *Epichloë festucae* (Lukito et al., 2019). In *F. fujikuroi*, loss of Set1 also resulted in increased production of bikaverin, fusarins and fusaric acid (Janevska et al., 2018b), thereby mirroring the Δ*ffkmt5* phenotype. Similarly, deletion of *CCL1* in *F. fujikuroi* and *F. graminearum* also resulted in an increased production of fusarins and bikaverin as well as zearalenone, respectively (Studt et al., 2017).

Less data is available for H3K36me3, though this histone PTM has been associated with SM biosynthesis in fusaria recently (Gu et al., 2017c; Janevska et al., 2018a). Notably, two proteins i.e., Set2 and Ash1, are associated with genome-wide H3K36me3 (Janevska et al., 2018a). While Set2 mediates H3K36 methylation in euchromatic regions, Ash1-mediated H3K36 methylation is predominately found in subtelomeric regions in *F. fujikuroi*. Loss of either resulted in decreased GA_3 but elevated fusarin and fusaric acid biosynthesis. Thus, strains deficient for H3K36me3 (Δ*set2* and Δ*ash1*) also result in reduced GA_3 levels but increased production of fusarins and fusaric acid in *F. fujikuroi* (Janevska et al., 2018a, 2018b; Studt et al., 2017), thereby again mirroring Δ*ffkmt5* phenotype. Future research is required to elucidate the genome-wide positioning of H4K20me and to unravel its relationship with H3K4me3 and H3K36me3.

4.3. Lack of Kmt5 induces MSB- and osmotic stress tolerance in both fusaria

We found strains lacking Kmt5 more tolerant towards oxidative (MSB) stress in *F. fujikuroi* and *F. graminearum*. In the case of *F. fujikuroi*, overexpression of FfKmt5 reversed this phenotype on MSB-supplemented media resulting in a more sensitive phenotype. No growth changes were induced when H_2O_2 was used as an oxidative agent in both fusaria. Recently, Shao et al. (2019) showed that H_2O_2 and MSB trigger different transcriptional pathways in *A. oryzae*. Generally, oxidative stress induces antioxidant enzymes including catalase (CAT), glutathione peroxidase (GPX) and superoxide dismutase (SOD) as well as the transcription factors Yap1 and Skn7 (Breitenbach et al., 2015; Lee et al., 1999; Morgan et al., 1997). In *A. oryzae*, different sets of CAT and SOD genes are activated by either H_2O_2 or MSB (Shao et al., 2019). Further, MSB treatment increased GPX expression whereas H_2O_2 rather triggered Yap1 transcription factors. Notably, different Skn7 transcription factors responded specifically either to H_2O_2 or MSB induced stress (Shao et al., 2019). Thus, the observed diverging tolerance induced by H_2O_2 and MSB stress of our *KMT5* mutants might be owned from the different transcriptional response of oxidatively regulated genes. In addition to the increased MSB tolerance, lack of *KMT5* resulted also in a higher tolerance to osmotic stress (NaCl and sorbitol) in both fusaria. In *F. fujikuroi*, overexpression of *KMT5* reversed this phenotype resulting in strains that are more sensitive towards the osmotic stress (sorbitol). If the increased stress tolerance of the *KMT5* deletion mutants results from alterations in DNA damage repair or through deregulation from stress-associated genes originally silenced by Kmt5 remains to be determined. The Hog1 pathway responds to osmotic stress but also contributes substantially to oxidative defence (de la Torre-Ruiz et al., 2015), and loss of the orthologue Hog1 i.e., *FfSak1* and *FgOS-2* resulted in mutants more sensitive to osmotic stress in *F. fujikuroi* and *F. graminearum*, respectively (Van Nguyen et al., 2012; Zheng et al., 2012). Thus, it is tempting to speculate that genes in this signal cascade are affected by Kmt5. A crosstalk between H4K20 and H3K9 methylation was shown to be not only fundamental for pericentric heterochromatin formation but also for DNA damage repair in higher eukaryotes (Kovaříková et al., 2018; Schotta et al., 2004). Schotta et al. (2004) demonstrated that pericentric H4K20me3 is severely reduced in *Su(var)3–9* mutants. Further, studies in mammalian cells suggest a co-regulatory function between H3K9me3, H4K20me3 and 53BP1 (Kovaříková et al., 2018). It remains to be determined whether a similar crosstalk is happening also in *Fusarium*, but it is noteworthy that loss of the H3K9 methyltransferase Dim5 led to an increased tolerance towards osmotic stress in *F. verticillioides*, a phenotype which is attributed to a hyper-phosphorylation of Hog1 in Δ*fvdim5* mutants (Gu et al., 2017a).

5. Conclusion

In this study, we have identified the histone methyltransferase Kmt5 that is solely responsible for mono-, di- and trimethylation of H4K20 in the two plant-pathogenic fungi *F. fujikuroi* and *F. graminearum*. Phenotypic characterisation of deletion as well as overexpression strains showed that Kmt5 is not essential for development and pathogenicity in both fusaria. While production of some SMs in *F. fujikuroi* and *F. graminearum* appear to rely on H4K20 methylation levels, others remain unaffected. Notably, observed findings are more likely to result from

secondary effects and not by Kmt5 directly targeting SM genes. Most strikingly, loss of Kmt5 resulted in increased tolerance to MSB and osmotic (NaCl and sorbitol) stress in both fusaria. Thus, Kmt5 is largely dispensable for basic metabolic and developmental processes in *F. fujikuroi* and *F. graminearum* but involved in MSB and osmotic stress response. Future studies on the genome-wide positioning of H4K20me3 will allow us to shed further light on the importance of this histone PTM in *Fusarium* spp.

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CRediT authorship contribution statement

Simone Bachleitner: Methodology, Investigation, Validation, Formal analysis, Visualization, Writing - original draft. **Michael Sulyok:** Investigation, Resources, Writing - review & editing. **Jens Laurids Sørensen:** Investigation, Resources, Writing - review & editing. **Joseph Strauss:** Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition. **Lena Studt:** Conceptualization, Methodology, Resources, Investigation, Supervision, Writing - original draft, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fgb.2021.103602.

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